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Synthesis of an ochre suppressor tRNA gene and expression in mammalian cells

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We have used site-specific mutagenesis to change the anticodon of a *Xenopus laevis* tyrosine tRNA gene so that it would recognize ochre codons. This tRNA gene is expressed when amplified in monkey cells as part of a SV40 recombinant and efficiently suppresses termination at both the ochre codon separating the adenovirus 2 hexon gene from a 23-kd downstream gene and the ochre codon at the end of the NS1 gene of influenza virus A/Tex/1/68. Termination at an amber codon of a NS1 gene of another influenza virus strain was not suppressed by the (Su$^+$) ochre gene suggesting that in mammalian cells amber codons are not recognized by ochre suppressor tRNAs. Finally, microinjection into mammalian cells of both (Su$^+$) ochre tRNA genes and selectible genes containing ochre nonsense mutations gives rise to colonies under selective conditions. We conclude that it should be possible to isolate a wide assortment of mammalian cell lines with ochre suppressor activity.

Key words: tRNA^{Tyr} gene/ochre suppressor/site-specific mutagenesis/expression

Introduction

All three nonsense codons are used for termination of translation in the genes of vertebrates and their viruses. There is a slight preference for ochre (UAA) codons in cellular genes and a strong bias against amber (UAG) codons as signals in viral genes (Kohli and Grosjean, 1981). Mutations within cellular genes that result in ochre and amber nonsense codons have been identified in some genetic diseases such as thalassemia (Chang and Kan, 1979). It is also possible that many of the variants isolated by selection of somatic cells are nonsense mutants (Capecchi *et al.*, 1977); however, short of isolating and sequencing the mutated gene this is difficult to establish. A few nonsense mutations in non-essential genes of mammalian viruses have been isolated (Grodzicker *et al.*, 1976; Gesteland *et al.*, 1977; Rawlins and Muzyczka, 1980; Cremer *et al.*, 1979), and, when studied, these mutants typically have < 1% wild-type activity (Summers *et al.*, 1983).

The use of nonsense mutations in systematic analysis of mammalian cell and viral genes awaits the development of cell lines containing functional suppressors. In both bacteria and yeast more suppressors of translation termination are mutant tRNA genes that recognize nonsense codons (Steege and Söll, 1979). In general, these Su$^+$ tRNA genes have anticodons complementary to nonsense codons. With the isolation of genes for tRNAs from vertebrates and the development of site-specific mutagenesis methods (Kudo *et al.*, 1981), it is now possible to design tRNA genes that might specify suppressor tRNAs. The stable integration of such tRNA genes should yield mammalian cell lines that are permissive for nonsense mutations.

Recently, we described the use of site-specific mutagenesis to change a *Xenopus laevis* tRNA^{Tyr} gene so that it would recognize amber (UAG) nonsense codons (Laski *et al.*, 1982b). This gene has amber suppressor activity when introduced into mammalian cells *via* an SV40 vector (Laski *et al.*, 1982b) or when stably integrated in the chromosome (Hudziak *et al.*, 1982). An interesting second class of suppressors would be those that recognize ochre nonsense codons. In this paper, we describe the generation of an ochre

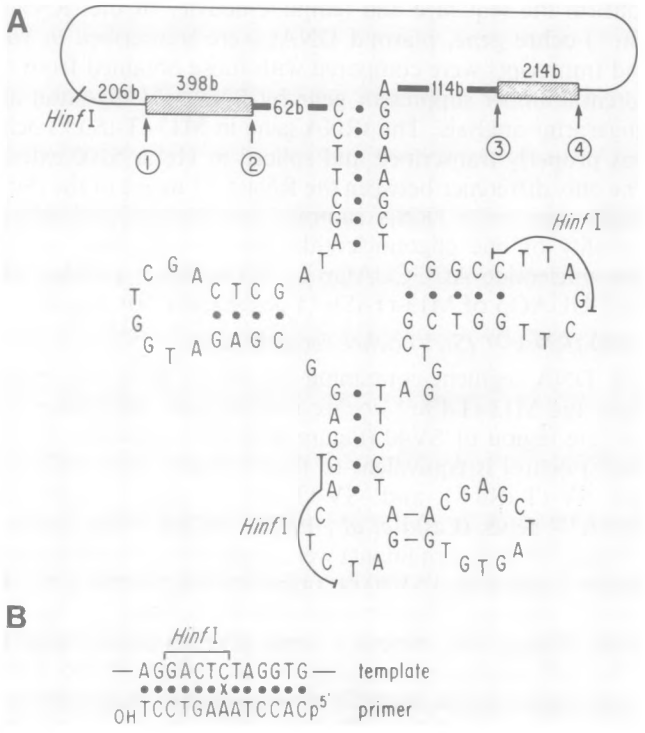

Fig. 1. (**A**) Structure of M13-tT-(Su$^+$) amber. M13-tT-(Su$^+$) amber contains a 263-bp *HaeII/HhaI* fragment of *X. laevis* DNA flanked by SV40 sequences cloned into the *HindIII* site of M13mp5. The tRNA^{Tyr} (Su$^+$) amber gene was constructed by the site-specific mutagenesis of the wild-type tRNA^{Tyr} gene (Laski *et al.*, 1982b). The sequence corresponding to the mature tRNA^{Tyr} (Muller and Clarkson, 1980) and its intervening sequence as well as the *HinfI* sites that cover and immediately flank the anticodon region are shown. For description of recombination sites see Laski *et al.* (1982b). (**B**) Sequence of the oligonucleotide primer. The oligonucleotide used for site-specific mutagenesis is complementary to the tRNA^{Tyr} (Su$^+$) amber gene except for one mismatch. Incorporation of the mutation results in an anticodon (UUA) complementary to an ochre codon and removes a *HinfI* site.

suppressor gene by site-specific mutagenesis of the same *X. laevis* tyrosine tRNA gene. This gene is transcribed into a functional tRNA which suppresses termination at ochre codons *in vivo*.

Results

Site-specific mutagenesis to generate a tRNA^{Tyr} (Su$^+$) ochre gene

The sequence of the *X. laevis* tRNA^{Tyr} (Su$^+$) amber gene is shown in Figure 1A. This gene had previously been isolated by primer-directed mutagenesis (Laski, *et al.*, 1982b) of the wild-type tRNA^{Tyr} gene. An ochre suppressor tRNA gene was generated by using a 13 base oligonucleotide in site-specific mutagenesis of the first position of the anticodon (Laski *et al.*, 1982b). The single base mutation would change the anticodon to UUA which is complementary to the ochre termination codon, UAA, and destroy a *Hinf*I cleavage site in the gene (Figure 1B). The oligonucleotide was added as primer for DNA polymerase I (Klenow fragment) on the single-stranded DNA recombinant M13-tT-(Su$^+$) amber. After transfection of bacteria with covalently closed, double-stranded DNAs, recombinant RF DNA from plaques were screened for cleavage with *Hinf*I. RF DNA from one of 24 plaques did not contain the *Hinf*I site.

We have previously shown that the tRNA^{Tyr} (Su$^+$) amber gene was accurately transcribed and the precursor processed in S100 extracts of mammalian cells (Laski *et al.*, 1983). To confirm the sequence and template activity of the tRNA^{Tyr} (Su$^+$) ochre gene, plasmid DNAs were transcribed *in vitro* and transcripts were compared with those obtained from the parental amber suppressor gene by RNase T1 digestion and fingerprint analysis. The tRNA gene in M13-tT-(Su$^+$) ochre was properly transcribed and spliced in HeLa S100 extract. The only difference between the RNase T1 digest of the (Su$^+$) amber and ochre tRNA samples was the expected shift in mobility of one oligonucleotide due to replacement of the oligonucleotide ACUCUAGp of M13-tT-(Su$^+$) amber with ACUUUAGp of M13-tT-(Su$^+$) ochre (data not shown).

SV40-tRNA^{Tyr} (Su$^+$) ochre recombinant

The DNA segment containing the tRNA gene was excised from the M13-tT-(Su$^+$) ochre recombinant and cloned into the late region of SV40 (Figure 2A). This construct [SV-tT-(Su$^+$) ochre] is equivalent to that prepared previously with the SV-tT-(Su$^-$) and SV-tT-(Su$^+$) amber suppressor tRNA^{Tyr} genes (Laski *et al.*, 1982a, 1982b). Virus stocks of all three SV40 recombinants were prepared by co-transfection of CV-1 cells with a SV40 rat preproinsulin recombinant, SV-rINS-7, that has a deletion/substitution in the early region of SV40 (Figure 2A). About 1 week after co-transfection, the CV-1 cells underwent a cytopathic degeneration and a virus stock was prepared. Such SV40 virus stocks occasionally contain variants which have DNA deletions or rearrangements. To test for this, CV-1 cells were infected with the three virus stocks or wild-type SV40 and replicating DNA was labeled by addition of $^{32}PO_4$ from 46 to 56 h post-infection. Viral DNA was purified by the method of Hirt, digested with *Hind*III and the products were analyzed by agarose gel electrophoresis (Figure 2B). DNA from all three infections produced the expected set of fragments and in amounts similar to that of wild-type SV40 infection. This suggests that the recombinant virus stocks had high titers and that the presence of the ochre supressor tRNA gene did not significantly inhibit SV40 replication.

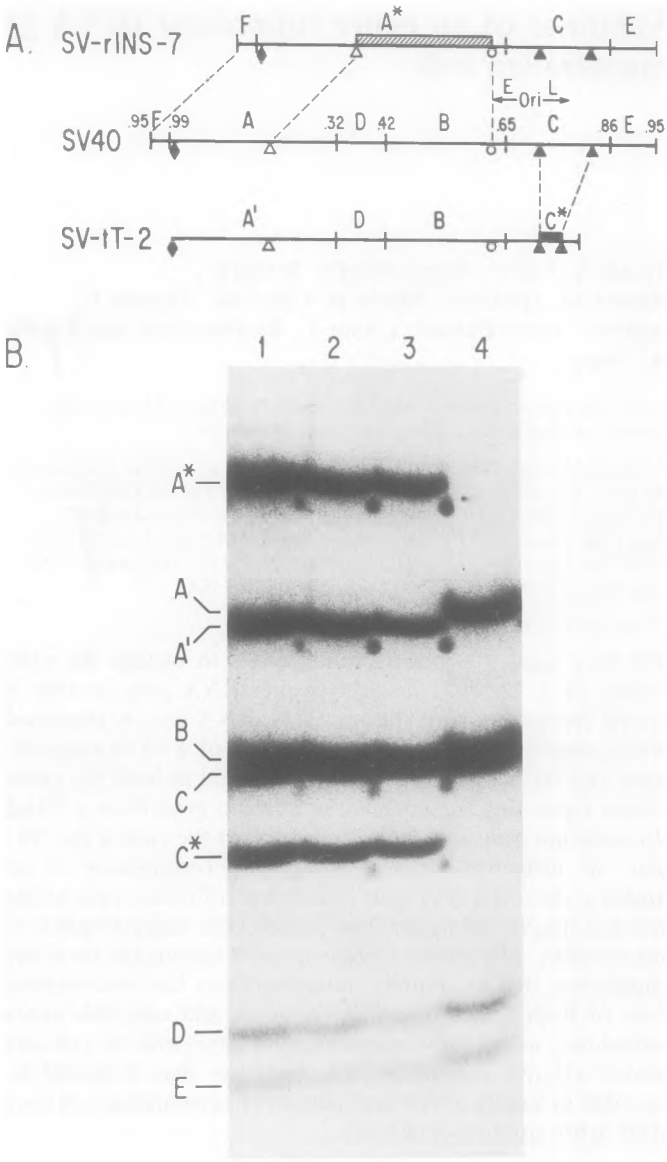

Fig. 2. (**A**) *Hind*III restriction map of SV-rINS-7, SV40, and SV-tT-2. SV-tT-2 represents the structure of the tRNA^{Tyr}-SV40 recombinants that were used to make the SV-tT-(Su$^-$), SV-tT-(Su$^+$) amber and SV-tT-(Su$^+$) ochre virus stocks. These tRNA^{Tyr}-SV40 recombinants are identical except for point mutations in the anticodon region of the tRNA^{Tyr} gene. Solid lines represent positions of the *Hind*III sites in SV40. SV-rINS-7 (Horowitz *et al.*, 1983) has an insertion of sequences from rat preproinsulin gene and a deletion of sequences from the early region of SV40 creating the new restriction fragment A* (Laski *et al.*, 1982a). SV-tT-2 has an insertion/deletion in the late region of SV40 creating the new restriction fragment C* and a deletion of late SV40 sequences (map unit 0.86 – 1.0) creating the new restriction fragment A'. The origin of replication (ori) and the direction of early and late transcription are shown. These recombinants have been previously described (Laski *et al.*, 1982a, 1982b). (**B**) Autoradiogram of *Hind*III digests of viral DNAs from SV-tT-(Su$^+$) amber-infected cells (**lane 1**), SV-tT-(Su$^+$) ochre-infected cells (**lane 2**), SV-tT-(Su$^-$)-infected cells (**lane 3**) and SV40-infected cells (**lane 4**). ^{32}P-Labeled viral DNA was digested with *Hind*III and the fragments were separated by electrophoresis on a 1.4% agarose gel. Bands are labeled as indicated in (**A**).

Expression of tRNA^{Tyr} (Su$^+$) ochre in mammalian cells

Cells infected with SV-tT-(Su$^+$) ochre, SV-tT-(Su$^-$), SV-tT-(Su$^+$) amber and SV40 were analyzed in parallel for production of tRNAs. In all cases, RNA labeled by addition of $^{32}PO_4$ between 46 and 56 h post-infection was resolved by electrophoresis in a 7.5% acrylamide/8.3 M urea gel (Figure 3). The wild-type tRNA^{Tyr} gene was efficiently expressed

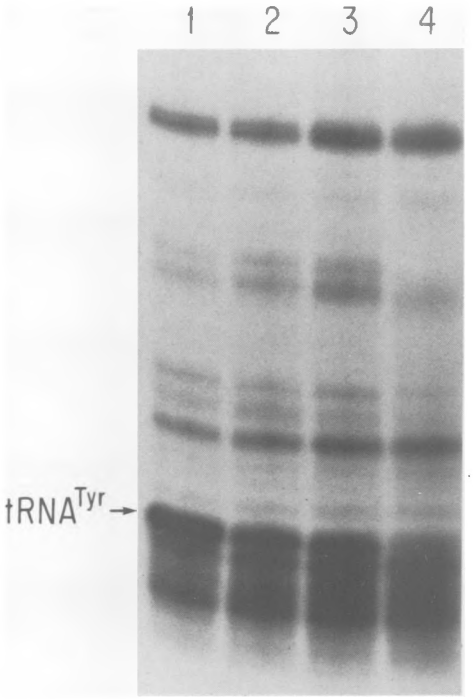

Fig. 3. Autoradiogram of [^{32}P]RNA isolated from CV-1 cells and analyzed on a 7.5% polyacrylamide/8.3 M urea gel. Cells were infected with 0.2 ml of SV-tT-(Su$^-$) (**lane 1**); with 0.2 ml of SV-tT-(Su$^+$) ochre (**lane 2**); with 0.2 ml of SV-tT-(Su$^+$) amber (**lane 3**); and with 0.1 ml of SV40 (10^8 p.f.u./ml) (**lane 4**). Arrow indicates location of mature tRNA^{Tyr}.

(lane 1) yielding a prominent band migrating as expected for a 76-nucleotide RNA. Compared with SV40-infected cells (lane 4), all three recombinant virus stocks yielded a prominent tRNA band (lanes 1–3). Both the tRNA^{Tyr} (Su$^+$) ochre (lane 2) and the tRNA^{Tyr} (Su$^+$) amber (lane 3) genes were expressed at ~20% of the level of the wild-type gene. For further analysis, radioactive RNA from the wild-type tRNA^{Tyr} and tRNA^{Tyr} (Su$^+$) ochre bands was eluted and purified by electrophoresis in a 10% polyacrylamide-4 M urea gel. RNAs from the major bands in the latter gel were digested with RNase T1 and analyzed by fingerprinting (Figure 4A and B). As anticipated a new oligonucleotide (ACUU*ψAm^{1}Gp) was present in the RNase T1 digest of (Su$^+$) ochre sample (Figure 4B) while two oligonucleotides ordinarily found in the wild-type tRNA^{Tyr} sample (ACUGp and ψAm^{1}Gp) (Figure 4A) were missing. The identity of the new oligonucleotide was confirmed by secondary digestion with RNase T2 followed by 2-dimensional t.l.c. analysis of the products (Figure 5). The RNase T2 digest contains the nucleotides Ap, Cp, Up, ψp, m^{1}Gp and an unidentified nucleotide designated U*p. U*p is probably a modification of the U located in the first position of the anticodon.

The modified nucleotide U*p was consistently present in low molar yields in the RNase T2 digestions (Figure 5). This is at least due partially to streaking in the second dimension during t.l.c. In addition, U*p is possibly labile with the degradation product co-migrating with Up.

Suppression of termination at UAA codons by tRNA^{Tyr} (Su$^+$) ochre

Suppression of termination of adenovirus hexon ochre codon. The most abundant adenovirus virion component is the hexon protein (polypeptide II). This 120 000 dalton protein is encoded by sequences that terminate in a single ochre (UAA) codon which is followed by a sequence of 648 bp of

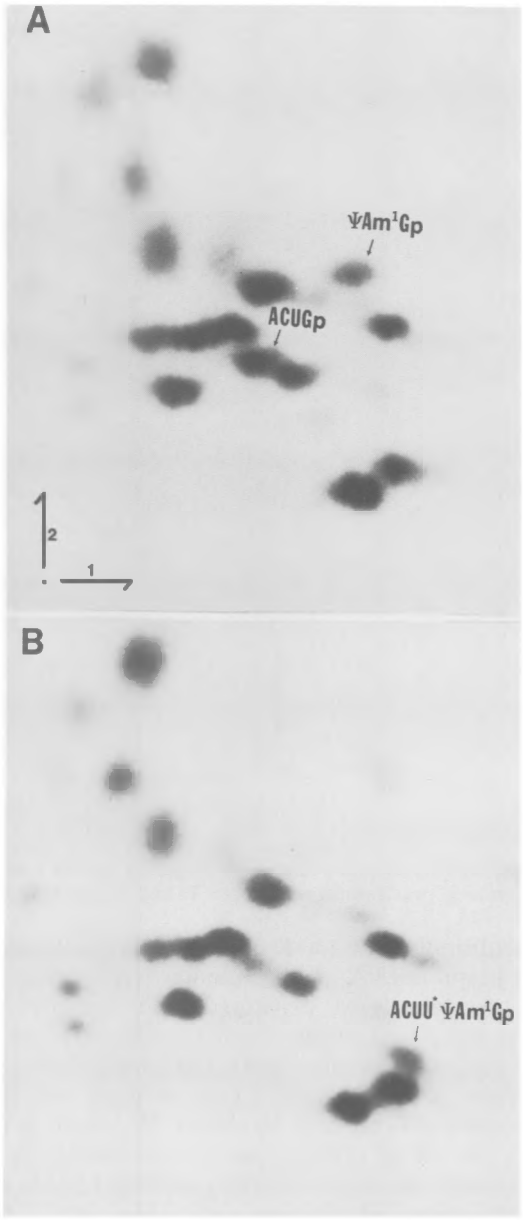

Fig. 4. RNase T1 fingerprints of ^{32}P-labeled tRNA^{Tyr} purified from SV-tT-(Su$^-$) (**panel A**) and SV-tT-(Su$^+$) ochre (**panel B**) infected cells. Radioactive tRNA^{Tyr} was eluted from bands indicated by the arrow in Figure 3, further purified by electrophoresis in a 10% polyacrylamide/4 M urea gel, and analyzed by RNase T1 mapping (Laski *et al.*, 1982b).

open reading frame (Figure 6B; see Akusjarvi *et al.*, 1981). The latter open reading frame is thought to encode a protease of 23 000 daltons which is found in the virion (Weber, 1976). Suppression of termination at the single ochre codon would produce a 140 000 dalton fusion protein that would be easy to detect since it would be the largest viral specified polypeptide.

To test for suppression of termination at ochre codons *in vivo*, CV-1 cells were infected with either SV-tT-(Su$^-$), SV-tT-(Su$^+$) amber, or SV-tT-(Su$^+$) ochre virus and incubated at 37°C for 24 h to permit synthesis of viral encoded products, including tRNAs. These cultures were then infected with Ad2 and, 24 h later, cells were labeled with [^{35}S]-methionine for 1 h. Gel analysis of total proteins extracted from the Ad2-infected cells is shown in Figure 6A (lanes 1–5). The anticipated 140 000 dalton protein was only synthesized when cells were co-infected with Ad2 and an SV-tT-(Su$^+$) ochre (lane 4).

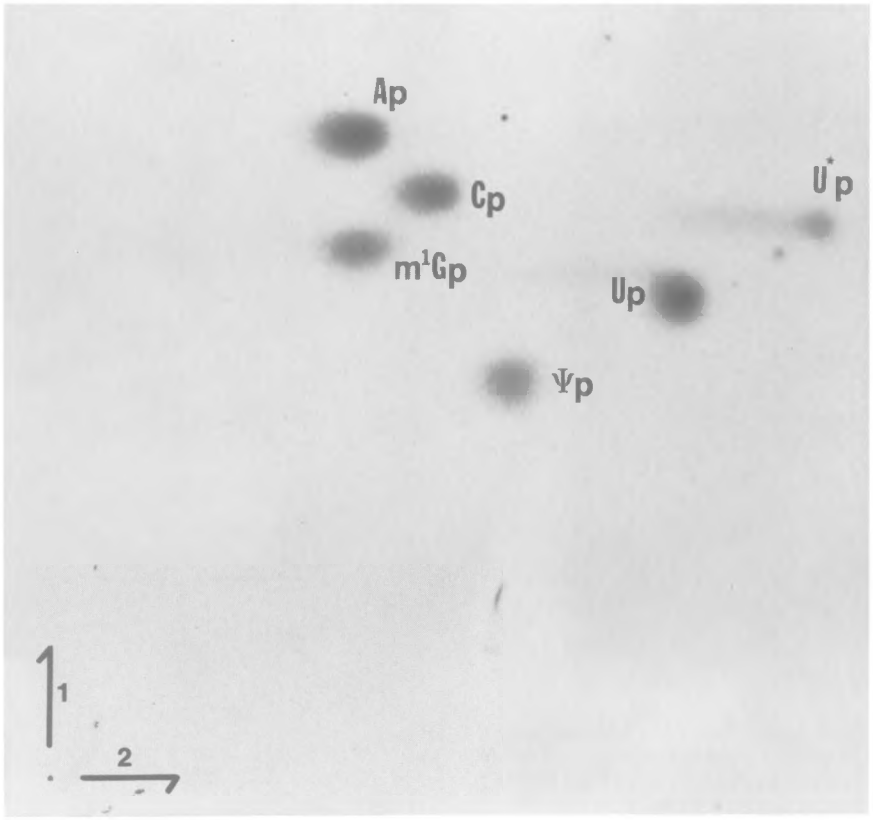

Fig. 5. Nucleotide composition analysis of RNase T1 product from ^{32}P-labeled tRNA^{Tyr} (Su$^+$) ochre. The oligonucleotide designated ACUU*ψAm¹Gp in Figure 4, panel B, was digested with RNase T2 and the products separated by 2-dimensional t.l.c. (Laski *et al.*, 1982b).

To confirm that the 140-K protein was a readthrough product of hexon mRNA, [^{35}S]methionine-labeled protein from the co-infected cells was denatured and immunoprecipitated with a guinea pig antiserum directed against denatured hexon protein (antiserum supplied by C. Gambkle and W. Deppert; see Cepko *et al.*, 1981). Gel analysis of the immunoprecipitated proteins is shown in Figure 6A (lanes 6–10). The 140-K protein was immunoprecipitated quantitatively with the hexon antiserum and must be the product of suppression of termination at the UAA codon of hexon mRNA. The efficiency of suppression was estimated as 10% by measuring the relative amount of label in the 140-K readthrough protein and the 120-K hexon protein. Interestingly, the 140-K readthrough protein was also immunoprecipitated by a monoclonal antibody specific for the group antigen of hexon trimers (Cepko and Sharp, 1982). This reactivity suggests that trimer formation of hexon is not precluded by the presence of the extra 23 kd of polypeptide.

Suppression of termination of influenza NS1 ochre codon. Different strains of influenza virus encode different size NS1 polypeptides. The sequences of a number of NS1 genes have been determined and show that translation of this polypeptide terminates at opal, ochre or amber codons in certain strains (Krystal *et al.*, 1983; Baez *et al.*, 1980; Parvin *et al.*, 1983). We have previously used a strain (A/CAM/46) with an amber codon to quantitate the level of suppression with the SV-tT-(Su$^+$) amber recombinant (Young *et al.*, 1983a). The NS1 gene of influenza A/Tex/1/68 virus terminates in an ochre (UAA) codon and readthrough at this codon should result in a product which is 17 amino acids longer than the 220 amino acid normal protein (Parvin *et al.*, 1983). To test further the suppressor activity of the SV-tT-(Su$^+$) ochre recombinant, CV-1 cells were infected with this virus stock and superinfected with influenza A/Tex/1/68 virus. The labeled cell extract was separated by electrophoresis on polyacrylamide gels (Figure 7, lane 3) and its protein pattern was compared with that of cells infected only with A/Tex/1/68 virus (lane 5). A readthrough polypeptide of the anticipated mol. wt. was observed only in extracts of cells co-infected with the (Su$^+$) ochre virus (lane 3 indicated by arrow) but not in control CV-1 cells (lane 5) nor in cells that either had previously been infected with the (Su$^-$) SV40 recombinant, or the (Su$^+$) amber virus (lanes 6 and 7, respectively; Table I). The last result shows that, as expected, the amber suppressor activity did not suppress termination at ochre codons. Control experiments showed that SV-tT-(Su$^+$) amber virus did suppress termination at the amber codon of the A/CAM/46 virus NS1 gene (lane 8).

The identity of the NS1 readthrough products following amber or ochre suppression was confirmed by immunoprecipitation using a monospecific rabbit antiserum (Young *et al.*, 1983b) made against NS1 protein synthesized in bacteria (data not shown). Optimal readthrough using the (Su$^+$) ochre virus was found to occur if influenza virus infection followed a 36–48 h pre-infection with the SV40 recombinant (data not shown). Use of preparations of the (Su$^+$) ochre virus and the (Su$^+$) amber virus at a 2-fold concentration did not increase the level of readthrough (Figure 7, lanes 4 and 7, respectively).

In bacterial systems, ochre supressors recognize both amber and ochre codons (Brenner and Beckwith, 1965). It was thus of interest to test whether the SV-tT-(Su$^+$) ochre virus would suppress termination at amber codons in mammalian cells. Pre-infection of cells with the (Su$^+$) ochre virus

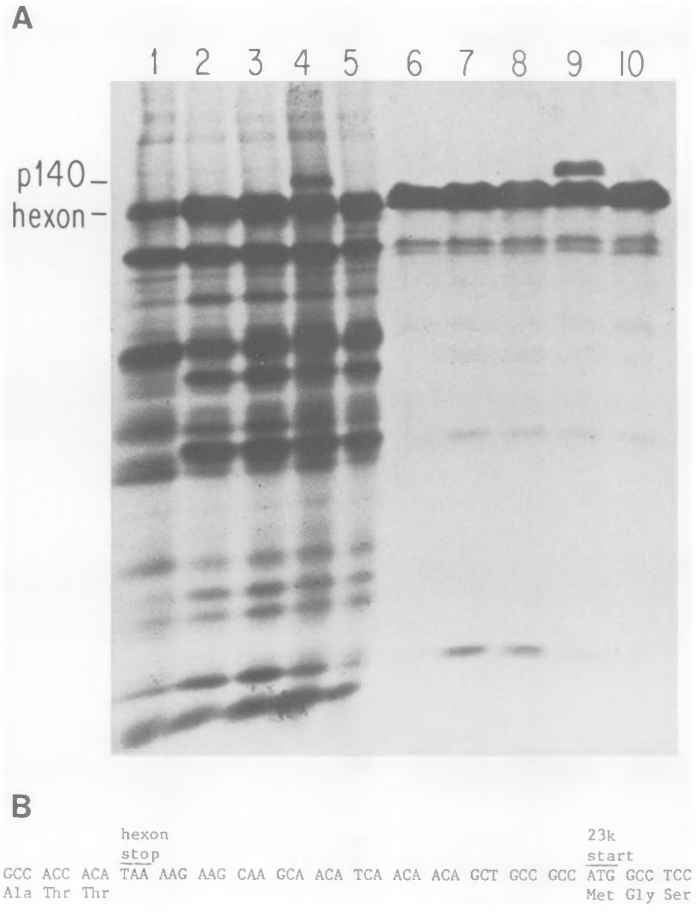

Fig. 6. Suppressor activity of SV40 (Su^+) ochre recombinant virus-infected cells as assayed by readthrough of adenovirus hexon mRNA. (**A**) Electrophoretic analysis on a 10% polyacrylamide gel [^{35}S]methionine-labeled proteins in CV-1 cells after infection with SV40 recombinants followed by Ad2 (Cepko *et al.*, 1981; Laemmli, 1970). CV-1 cells were infected with 0.2 ml of SV-tT-(Su^-) or 0.2 ml of SV-tT-(Su^+) amber or 0.2 ml of SV-tT-(Su^+) ochre or 0.1 ml of SV40 (10^8 p.f.u/ml) or mock infected. 24 h later, cells were further infected with Ad2 at a m.o.i. of 100. 24 h later, cells were labeled with 100 μCi of [^{35}S]methionine for 1 h. Cells were lysed in 1 ml of RIPA buffer (Cepko *et al.*, 1981) and proteins were either directly analyzed by electrophoresis (**lanes 1–5**) or were first immunoprecipitated with an antibody directed against denatured hexon protein of Ad2 (Cepko and Sharp, 1982) (**lanes 6–10**). **Lanes: 1** and **6**, mock infection followed by Ad2; **2** and **7**, SV40 followed by Ad2; **3** and **8**, SV-tT-(Su^-) followed by Ad2; **4** and **9**, SV-tT-(Su^+) ochre followed by Ad2; **5** and **10**, SV-tT-(Su^+) amber followed by Ad2. (**B**) Sequence surrounding Ad2 hexon stop codon and methionine initiation site of 23-K polypeptide (Akusjarvi *et al.*, 1981).

did not result in a readthrough of the amber codon of the NS1 gene of A/CAM/46 virus (Table I). As mentioned above, under identical conditions, the SV-tT-(Su^+) amber virus did suppress termination of the NS1 gene of A/CAM/46 virus and the (Su^+) ochre virus was active in suppression of the equivalent gene of A/Tex/1/68 virus.

Selection of cell lines containing ochre suppressor activity

We have previously described the isolation of amber nonsense mutations in the bacterial Tn5 kanamycin-resistant gene (NPT-II) and the Herpes simplex 1 thymidine kinase gene (HSV-tk) (Hudziak *et al.*, 1982). These mutations were identified and characterized by expressing the two genes in *Escherichia coli*. Similar methods were used to isolate ochre nonsense mutations in the same two genes. In both cases, a bacterial $tRNA^{Tyr}$ (Su^+) ochre gene gave efficient suppression. To facilitate expression in mammalian cells, the NPTII$^-$ ochre gene was inserted downstream of the long terminal repeat element of avian sarcoma virus while the HSV-tk$^-$ ochre gene was used with its natural promoter (Hudziak *et al.*, 1982). These genes provide a biological selection for ochre suppressor activity in tk$^-$ cells.

Microinjection was used to introduce efficiently combinations of cloned genes into cells. Combinations of the NPTII$^-$ and HSV-tk$^-$ ochre genes and (Su^+) ochre or (Su^-) $tRNA^{Tyr}$ genes were microinjected into 1000 LMtk$^-$ cells to test whether cell lines could be established by selection for suppression of the ochre mutations. Co-injection of HSV-tk and NPTII wild-type genes with the *X. laevis* (Su^-) tRNA gene and subsequent selection for either G418 resistance or in HAT media resulted in ~20% of the cells giving rise to colonies (experiment C of Table II). When the HSV-tk$^-$ and NPTII$^-$ ochre mutants were microinjected under the same conditions, no colonies were obtained with either selection (experiment B of Table II). However, injection of the *X. laevis* (Su^+) ochre gene with the two nonsense mutants yielded ~50 colonies (experiment A of Table II) which is approximately one-quarter of that obtained with wild-type genes. These results strongly suggest that mammalian cell lines with biologically significant levels of ochre suppressor activity can be propagated.

Discussion

Mammalian cells are non-permissive for translation of all three types of nonsense codons. Selection of cell lines with suppressor activity by conventional methods of reversion of phenotype of nonsense mutants has so far not been successful. An alternative approach that we have chosen is the transformation of cells with tRNA genes with suppressor activity. Site-specific mutagenesis has been used to alter the anticodon of a *X. laevis* tyrosine tRNA gene so that it would recognize an ochre (UAA) codon. This gene is efficiently expressed as mature spliced tRNA when introduced into cells as part of a replicating SV40 recombinant. We have previously constructed an amber suppressor using the same *X. laevis* tyrosine tRNA gene (Laski *et al.*, 1982b). The amber and ochre (Su^+) genes gave comparable levels of suppression of their specific nonsense codons in CV-1 cells.

Suppression activity at ochre codons was assayed using two different viruses. In both cases, the (Su^+) ochre tRNA gene was introduced into cells as part of a replicating SV40 recombinant before infection by a second virus. This results in the synthesis of high levels of (Su^+) tRNA and probably establishes near optimal conditions for suppression at nonsense codons. Co-infection of these cells with Ad2 results in the synthesis of a novel 140 000 dalton fusion protein due to partial suppression of termination at the single ochre codon between the reading frames encoding the hexon (120 000) and protease (23 000) polypeptides (Akusjarvi *et al.*, 1981). Assuming that the readthrough product was stable during the pulse labeling period, the efficiency of suppression was ~10%. The level of suppression of termination at the ochre codon of the NS1 gene of influenza virus A/Tex/1/68 was also examined. Cells pre-infected with the (Su^+) ochre SV40 recombinant synthesized a readthrough polypeptide at a 30% efficiency. This level was comparable with that obtained under equivalent conditions with (Su^+) amber SV40 recombinant and another strain of influenza virus which terminates its NS1 gene with an amber codon (Young *et al.*, 1983a). Thus amber and ochre codons are probably equally

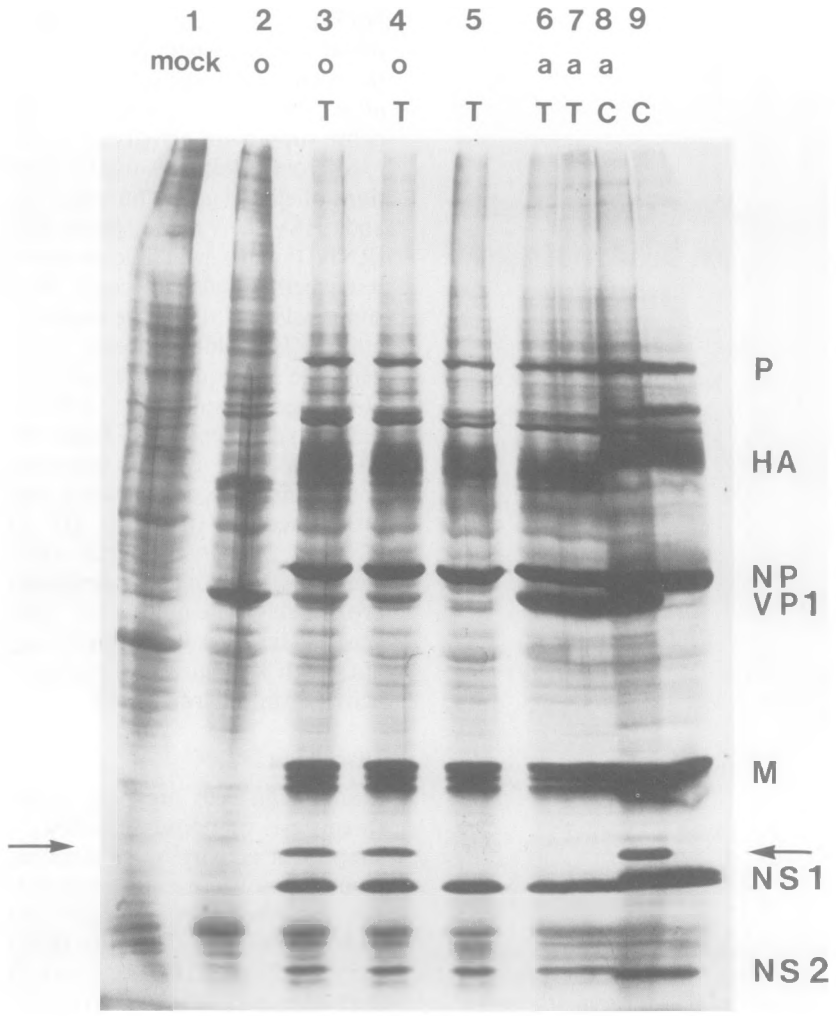

Fig. 7. Suppressor tRNA activity of SV40 (Su^+) ochre recombinant virus-infected cells as assayed by readthrough of influenza virus NS1 mRNA. CV-1 cells (1 x 10^6 cells) were infected with SV-tT-(Su^+) ochre virus or SV-tT-(Su^+) amber virus as indicated. 36 h later cells were superinfected with influenza virus A/CAM/46 or A/Tex/1/68 virus (m.o.i. = 10). After another 6 h, cells were pulse-labeled using [^{35}S]methionine and cell extracts were subjected to electrophoresis on a gradient polyacrylamide gel as previously described (Young *et al.*, 1983a). **Lanes: 1**, uninfected cells; **2**, cells infected for 42 h with SV-tT-(Su^+) ochre virus; **3**, cells were first infected for 36 h with (Su^+) ochre virus and then superinfected for 6 h with influenza A/Tex/1/68 virus; **4**, same as **lane 3** except that concentration of SV-tT-(Su^+) ochre virus was 2-fold higher; **5**, cells infected for 6 h with A/Tex/1/68 virus; **6**, cells were first infected for 36 h with (Su^+) amber virus (Laski *et al.*, 1982b) and then superinfected for 6 h with influenza A/Tex/1/68 virus; **7**, same as **lane 6** except that concentration of SV-tT-(Su^+) amber virus was 2-fold higher; **8**, cells were first infected for 36 h with (Su^+) amber virus and then superinfected for 6 h with influenza A/CAM/46 virus; **9**, cells infected for 6 h with A/CAM/46 virus. 'O' and 'a' indicate the (Su^+) ochre and the (Su^+) amber SV40 recombinant preparations, respectively. 'T' and 'C' identify influenza A/Tex/1/68 and A/CAM/46 virus infected cells, respectively. The mol. wts. of the P, HA, NP, M1, NS1 and NS2 polypeptides are 82–86 K, 77 K, 56 K, 28 K, 23 K and 14 K, respectively. The arrows indicate the positions of the NS1 readthrough products of A/Tex/1/68 and A/CAM/46 viruses, respectively.

susceptible to suppression by tRNAs in mammalian cells. Detailed studies of suppression of nonsense codons in bacterial genes have revealed that the sequence context of the codon is important in specifying the efficiency of readthrough (Bossi and Roth, 1980; Miller and Albertini, 1983; Bossi, 1983). It is difficult to judge the effect of context on the levels of suppression of the Ad2 hexon and influenza virus NS1 genes; however, it is possible that some ochre codons might be more efficiently suppressed under comparable conditions. In any case, a 30% level of readthrough is typical of strong yeast suppressors and adequate for most genetic studies (Piper, 1979).

In *E. coli*, ochre suppressors recognize both amber and ochre codons (Brenner and Beckwith, 1965) in accordance with the wobble hypothesis of Crick (1966). This is not the case in yeast where ochre tRNA suppressors are specific for ochre codons (Gilmore *et al.*, 1971). This specificity is thought to be due to the modification of the U in the first position of the UUA anticodon to a base which cannot recognize amber codons by wobble pairing (Piper, 1979). The *X. laevis* $tRNA^{Tyr}$ (Su^+) ochre gene is also specific for ochre codons. Pre-infection of CV-1 cells with an SV40 recombinant containing this gene did not result in suppression of termination at an amber codon of the NS1 gene. Analysis of purified (Su^+) ochre $tRNA^{Tyr}$ also revealed a modification in its anticodon loop, probably at the first position of the anticodon. The chemical nature of this modification has not been determined; however, its presence is consistent with the observed specificity.

The study of both viral and cellular genetics in mammalian systems would greatly benefit from the availability of efficient nonsense suppressors. Stable cell lines expressing amber suppressor activity have been isolated (Hudziak *et al.*, 1982). These initial lines suppress termination at a 5% level (Young *et al.*, 1983a; M. Capecchi, unpublished data) and it is not clear whether higher levels of suppression would have a

Table I. Suppression of termination at NS1 ochre or amber codons

Conditions	% Suppression	
	A/Tex/1/68 NS1 (ochre)	A/CAM/46 SN1 (amber)
CV-1	< 1	< 1
CV-1 infected with (Su^+) ochre SV40 recombinant	31.3	< 1
CV-1 infected with (Su^+) amber SV40 recombinant	< 1	32
CV-1 infected with (Su^-) SV40 recombinant	< 1	< 1

SV40 recombinants expressing suppressor tRNA genes were used to infect CV-1 cells and the amount of ochre and amber suppression was determined by quantitating the readthrough product of the NS1 polypeptide of influenza A/Tex/1/68 and A/CAM/46 virus, respectively (Figure 7). (% suppression represents the amount of readthrough product of NS1 divided by the amount of NS1 and of readthrough product of NS1.) Conditions for infection of CV-1 cells were as described for Figure 7. Suppression values represent the average of two independent experiments.

Table II. Transformation frequency obtained by co-injecting *X. laevis* ochre suppressor or wild-type tyrosine tRNA gene with HSV-tk and NPTII ochre mutants

Genes injected	Selected on G418	Selected on HAT
A. HSV-tk^- (ochre) NPTII$^-$ (ochre) *X. laevis* ochre suppressor tRNA	58/1000	44/1000
B. HSV-tk^- (ochre) NPTII$^-$ (ochre) *X. laevis* wild-type tRNA	0/1000	0/1000
C. HSV-tk (wild-type) NPTII (wild type) *X. laevis* wild-type tRNA	211/1000	196/1000

A sample containing ~ 10 copies/cell of each gene was injected into LMtk$^-$ cells (Hudziak *et al.*, 1982). The cells were then cultured for 24 h in non-selective medium to allow for expression of the injected genes. Following this incubation period, the cells were transferred into either HAT medium or medium containing G418 (300 μg/ml) to select for either tk^+ or NPTII$^+$ phenotypes, respectively. After 3 weeks in selective medium, each culture dish (a total of 150) were scored for the presence of healthy colonies.

deleterious effect on cellular physiology. Analysis of the sequence of a number of mammalian genes shows that the first nonsense codon in a gene is typically followed by a second nonsense codon at an interval suggesting a random distribution (Kohli and Grosjean, 1981). Thus for a typical gene, only 16 additional residues are added upon suppression of termination. The presence of tandem termination codons (Fiers *et al.*, 1978; Reddy *et al.*, 1978) in SV40 explains the absence of shifts in polypeptide mol. wts. in infected cells expressing (Su^+) activity. In the case of adenovirus, the only shift in polypeptide synthesis observed in cells with ochre suppressing activity was the hexon-protease readthrough product. These results suggest that low and perhaps intermediate levels of ochre suppressor activity will probably be compatible with normal cell functions.

Efficient colony formation was observed after selection in either HAT medium or medium containing G418 after co-microinjection into tk^- L cells of HSV-tk^- and NPTII$^-$ ochre mutants, respectively, and the (Su^+) ochre gene. This strongly suggests that biologically significant levels of ochre suppressor activity are not toxic to cells. The availability of selectable markers with defined ochre mutations, tRNA genes with ochre suppressor activity, and efficient methods for introduction of these genes into mammalian cells should permit the isolation of an assortment of cell lines with ochre suppressor activity.

Materials and methods

The generation of M13-tT-(Su^+) ochre by site-specific mutagenesis

The oligonucleotide primer C-A-C-C-T-A-A-A-G-T-C-C-T was synthesized by using a modified phosphotriester method (Narang *et al.*, 1978 and references cited therein) and purified as a single peak by h.p.l.c. A 5′-terminal phosphate was added with T4 polynucleotide kinase. Viral DNA of M13-tT-(Su^+) amber was purified by alkaline sucrose sedimentation.

Primer-directed mutagenesis reactions were as described (Laski *et al.*, 1982b).

In vitro transcription and splicing reactions were performed with S100 extracts (Laski *et al.*, 1983). Analysis of RNase digestion products was performed as described (Laski *et al.*, 1982b).

Cloning of $tRNA^{Tyr}$ (Su^+) ochre into SV40

The segment containing the $tRNA^{Tyr}$ (Su^+) ochre gene from M13 was inserted into SV40 as described previously for M13-tT-(Su^-) and M13-tT-(Su^+) amber (Laski *et al.*, 1982b). These clones were designated pSV-tT-(Su^-), pSV-tT-(Su^+) amber and pSV-tT-(Su^+) ochre.

Virus and cell lines

Production of recombinant SV40 virus stocks and purification of DNA and RNA were by established procedures (Laski *et al.*, 1982a, 1982b).

Immunoprecipitation with monoclonal antibodies was previously described (Laski *et al.*, 1982b).

Influenza virus stocks were prepared and used as described previously (Young *et al.*, 1983a).

The methodology used to isolate and characterize ochre nonsense mutations in the Tn5 kanamycin resistance gene (NPT-II) and the HSV-tk gene has been described previously (Hudziak *et al.*, 1982). The techniques for microinjection and selection were also described previously (Hudziak *et al.*, 1982).

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